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# Some Observations on the Biology of the Cholera Spirillum

BY

Wm. B. Wherry, M. D.

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# LETTERS OF TRANSMITTAL.

DEPARTMENT OF THE INTERIOR,
BUREAU OF GOVERNMENT LABORATORIES,
OFFICE OF THE SUPERINTENDENT OF LABORATORIES,
*Manila, September 20, 1904.*

SIR: I have the honor to transmit herewith a paper by Dr. Wm. B. Wherry of the Biological Laboratory on "Some Observations on the Biology of the Cholera Spirillum."

I am, very respectfully,

PAUL C. FREER,
*Superintendent Government Laboratories.*

Hon. DEAN C. WORCESTER,
*Secretary of the Interior, Manila, P. I.*

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DEPARTMENT OF THE INTERIOR,
BUREAU OF GOVERNMENT LABORATORIES,
BIOLOGICAL LABORATORY, OFFICE OF THE DIRECTOR,
*Manila, August 31, 1904.*

SIR: I have the honor to transmit herewith and recommend for publication "Some Observations on the Biology of the Cholera Spirillum," by Wm. B. Wherry, M. D., Bacteriologist Biological Laboratory.

Very respectfully,

RICHARD P. STRONG,
*Director Biological Laboratory.*

Dr. PAUL C. FREER,
*Superintendent Government Laboratories, Manila, P. I.*

# SOME OBSERVATIONS ON THE BIOLOGY OF THE CHOLERA SPIRILLUM.

By Wm. B. Wherry, M. D., *Bacteriologist, Biological Laboratory.*

## INTRODUCTION.

The following observations were made during the past year while I was engaged in some studies preliminary to and in connection with the subject of toxin production.

Such marked variations in the morphology and biochemical characters of the cholera spirillum occurred during some earlier work that it was deemed advisable to adopt a modification of the methods of standardizing culture media, published as the "Procedures," etc., by the Committee of American Bacteriologists.[1]

It is to be regretted, from a purely descriptive standpoint, that the organisms were not grown upon media prepared exactly according to the recommendations of the American Committee; but, since the main issue concerned the factors influencing toxin production, and since it was impossible to carry on two entirely separate sets of observations, a slight modification of these recommendations was employed for reasons which are given below.

Notwithstanding the use of these methods, a comparison of the biochemical peculiarities of the cultures chosen for this study reveals many points of difference between them—such as the production of a pellicle on bouillon by one, while another gives a diffuse cloudiness, the presence or absence of the cholera-red reaction, variations in the growth on potato, or in the type of the liquefaction of gelatin, etc. Many of these points of difference are

[1] *The Reports and Papers of the American Public Health Association*, 1898, XXIII, p. 60; or, for a brief summary of this report, *vide* L. Grimbert, on the Diagnosis of Bacteria by their Biochemical Functions, *Arch. d. Parasitologie*, 1903, VII, p. 304.

still emphasized in bacteriological literature, especially in the descriptions of single species. A careful preliminary study of one of these cholera cultures ("579") revealed such a wide variation in its morphology and in some of the details of its cultural characteristics, that I was forced to the conclusion that they could not be seriously considered in species description—since they are variations which will occur at intervals in the same culture.

It is hoped that this study, which was carried out under more uniform conditions than can be attained by older methods, will emphasize the variability of some bacteria and in a measure further the investigation of those factors entering into the production of such variations.

I have decided to present the subject-matter in the following order:

I. A description of the method of preparing and neutralizing the media.
II. The source, isolation, biochemical peculiarities, and varations of culture "579," with special reference to—
  (*a*) The demonstration of the cholera-red reaction.
  (*b*) The liquefaction of gelatin.
  (*c*) The optimum reaction.
  (*d*) The production of alkali.
III. A description of the source and isolation of five other cholera cultures and of their resemblance to one another, and to culture "579."
IV. Their growth in the presence of carbohydrates.
V. Their relationship as shown by agglutinating and bactericidal sera.
VI. Their pathogenicity.
VII. Their morphology and pleomorphism.
VIII. Summary and conclusions.

During this comparative study of a number of cultures from different sources, every precaution was taken to avoid contaminating one strain with portions from another culture, and the purity of each was controlled by frequent microscopical examinations and plating in gelatin or agar.

## I. A DESCRIPTION OF THE METHOD OF PREPARING THE MEDIA.

One of the chief modifications of the methods recommended in the "Procedures" concerns the way in which the media was neutralized and the desired reaction obtained. The recommended method consists, briefly, in titrating a portion of the medium, as near the

boiling point as possible, with phenolphthalein as an indicator; obtaining an accurate neutral point by the addition of normal sodium hydroxide to the bulk of the medium, and then adding sufficient normal hydrochloric acid to give the desired reaction. In this work the acidity or alkalinity to phenolphthalein was adjusted by the addition of normal sodium hydroxide alone, and unless otherwise stated the reaction refers to that established before sterilization. The term "final reaction" indicates the reaction of the medium after sterilization or just before its use. The sign (+) stands for acidity, while (—) indicates alkalinity. The figures are in per cent. Thus + 1 means 1 per cent[1] acid with phenolphthalein as an indicator (slightly alkaline to litmus).

This modification was adopted for the following reasons: Our *préparateur* made some bouillon in which the cholera spirillum would not grow at 35°–37°, whereas *B. coli* did so in it, luxuriantly, at that temperature. Upon investigating this phenomenon it was found that if, after neutralization with sodium hydroxide, the precipitated acid albumins were first filtered and then the hydrochloric added, the acid may exert a germicidal or inhibiting effect upon the cholera spirillum—the degree of inhibition apparently depending upon the thoroughness with which the acid albumins have been removed. Further, this inhibiting effect is more marked at body than at room temperature.

I have not been able to reproduce this phenomenon at every trial. Whether this is due to a variation in the amount of acid albumins actually removed on neutralization (it is well known that a portion of the precipitate formed by neutralization redissolves in an excess of alkali), or to some undetermined cause, the fact remains that such a fluid will sometimes completely inhibit the growth of the cholera spirillum, but not that of *B. coli*. It seems as if work with chemically pure solutions might furnish another biological proof of the existence of ionproteid compounds and also an instance of a *specific* (?) *antitoxic action exerted by a proteid.* This would not be the only example of such an antitoxic action, for, as shown by Kahlenberg and True,[2] hydrogen and various metallic

[1] One per cent acid or alkaline to phenolphthalein would, in chemical terms, represent 10 cubic centimeters of a normal acid or alkali to 1,000 cubic centimeters of the medium employed, or N/100.

[2] The Theory of Electrolytic Dissociation, H. C. Jones, 268–270. In my case, however, the action may possibly be due to free H ions from the hydrochloric acid, since it is a well-known fact that even small traces of acetic, hydrochloric, or citric acids completely destroy the growth of *Spr. cholerae.*

Kations exert a marked toxic action, at certain concentrations, on the germinating seedlings of *Lupinus albus*. The copper ion was found to be especially toxic and the seedlings barely lived in a solution containing a gram-molecular weight of copper ions in 51,200 liters of solution. But, when the copper ion was in combination with an organic complex, as in Fehling's solution, the roots would grow in a solution of this salt which contained a gram-atomic weight of copper in 400 liters.

The American committee does not recommend the removal of the acid albumins preliminary to the addition of the acid, but since Schultz-Schultzenstein [1] has shown that in fluids containing albumin or peptone or both, 0.097—0.217 per cent of HCL will destroy the cholera spirillum in an hour; and since further, as demonstrated by Smith,[2] hydrochloric acid is destructive to diphtheria toxin; and again, since Ritchie [3] has pointed out a similar destructive action upon tetanus toxin, it was deemed advisable to leave the hydrogen ion out of the media.

The method of sterilization indicated in the "Procedures" was followed with the exception that bouillon and agar were sterilized in the autoclave at 120° for half an hour. This prolonged sterilization at a high temperature was found necessary and expedient on account of some very resistant spored organism encountered during the last hot season. It does not noticeably affect the nutritive qualities of agar or bouillon, although the initial acidity is raised about 0.5 per cent. When — 1 agar or bouillon is subjected to such an autoclaving a precipitate is usually thrown down. On filtration and further autoclaving the alkalinity is found to have been diminished by about 0.5 per cent.

This discrepancy, according to the "Procedures," "is perhaps due to side reactions which are not understood." The usual formation of a precipitate, which seems to vary in proportion to the amount of alkali added, with the simultaneous reduction in the alkalinity of the medium, seems to point to the hydrolysis and separation of insoluble compounds under the influence of the alkali.

[1] Schultz-Schultzenstein: Zur Kenntnis der Einwirkung des menschlichen Magensekrets auf Choleravibrionen. *Cent. Bakt. 1st Abt.*, 1901, XXX, 785–790.

[2] Theobald Smith: The Relation of Dextrose to the Production of Toxine in Bouillon Cultures of the Diphtheria Bacillus. *Jour. of Exper. Med.*, 1899, IV, 383.

[3] J. Ritchie: Artificial Modifications of Toxins with Special Reference to Immunity. *Journal of Hygiene*, 1901, I, 130.

Contrary to a statement made in the footnote on page 72 of the "Procedures," I have several times noted the evolution of ammonia when bouillon is boiled after the addition of the fixed alkali. As might be expected, this is most marked in the case of fermented bouillon, as illustrated in the following instance:

Three thousand cubic centimeters of sugar-free bouillon had an initial reaction of + 3.0; 2.5. per cent (75 cubic centimeters) normal sodium hydroxide was added and thoroughly mixed. This should have given a calculated reaction of + 0.5. The solution, containing a dense flocculent precipitate, was boiled for two minutes. During ebullition an appreciable quantity of ammonia was given off which could be detected both by the odor and by the blueing of red litmus paper moistened with distilled water. The solution was filtered, brought up to 3,000 cubic centimeters by the addition of distilled water, and then showed a reaction of + 1.0—i. e., the acidity had been raised 0.5 per cent over the calculated one.

A similar change occurs in unfermented meat extract, but here the escape of volatile alkali takes place more slowly.

## II. THE SOURCE, ISOLATION, BIOCHEMICAL PECULIARITIES, AND VARIATIONS OF CULTURE "579."

### Necropsy No. 579.

Emeterio Darita, age 28 years, male, Filipino, residence, Lorcha *Horatio* (boat on the Pasig River). Died April 17, 1903, after an illness of ten hours. Clinical diagnosis, cholera. Autopsy performed three and three-fourths hours after death.

It was learned that the man had numerous stools and considerable vomiting before death.

The body was that of a very muscular man. There was not much shrivelling of the skin. The face was in repose. The feet were in extreme flexion. The fingers were in forced flexion and could not be straightened. The skin of the palms, the soles of the feet, the fingers, and the toes was shrivelled, the tissue beneath it being dry and comparatively bloodless. On the soles of the feet were a number of blister-like elevations, varying from about 2 to 20 millimeters in diameter. These were found to be perfectly dry on section. The white of the eyes was icteric. The muscles were very firm. On section the body tissues appeared abnormally dry. The superficial glands were not enlarged.

The *thoracic cavity* contained no fluid. There were a few rather firm, fibrinous adhesions between the lungs and the anterior thoracic walls. Posteriorly, the lower part of both lungs was bound down

by rather firm, fibrinous adhesions. The *thoracic organs* were covered by a scanty adhesive secretion.

The *lungs* were somewhat emphysematous throughout. The *pericardial cavity* contained a small amount of sticky secretion.

The *heart* was about normal in size and was in firm systole. It contained a considerable amount of thick, very dark, clotted blood. The blood which oozed from the larger arteries and coronary vessels was likewise of a very dark color and semicoagulated consistency. Otherwise the heart appeared normal. There was a persistent thymus gland about 5 by 15 millimeters in size.

The *peritoneal cavity* contained no fluid. The appendix was normal. The surface of the visceral and parietal peritoneum was covered with a scanty very adhesive secretion, which dried readily on exposure to the air. The parietal and visceral peritoneum throughout was of a rosy pink color.

The *spleen* was somewhat small in size and rather soft, and showed no particular changes on its surface or on section.

The *gall bladder* was filled with dark-green bile.

The *liver* was about normal size. The surface appeared somewhat cloudy, and on section the lobular markings were indistinct.

The *kidneys* were of about normal size; the capsules stripped readily, leaving a dark-red surface, on which the stellate veins stood out prominently. On section, the cortical and medullary markings were very indistinct.

The *stomach* contained a considerable amount of fluid substance, with many well-preserved rice granules. Its mucosa showed no particular change. The *intestinal tract* throughout contained a quantity of whitish, mucoid substance, which showed many white flocculi or rather flecks of a white albuminoid material. The secretion was very slippery while wet, but became very sticky on drying. The mucous surface of the duodenum and jejunum showed no change, but appeared rather whiter than normal. The mucosa of the upper two-thirds of the ileum showed no particular change, but a few of the solitary follicles were enlarged. In the lower third of the ileum the solitary follicles and Peyer's patches were quite generally enlarged, and some of the Peyer's patches appeared congested through the mucosa. The enlargement of the lymph follicles was most marked just above the ileo-cecal valve, where they were about 2 millimeters in diameter. In several places in the lower

portion of the ileum the mucosa was almost completely desquamated for a distance of several inches. The *pancreas* appeared rather pale on section.

The *mesenteric glands* were enlarged throughout to the size of almonds, and were pale on section (a common post-mortem finding here).

The *colon* and the upper portion of the *rectum* contained fluid contents similar to that described above. Here the mucous surface showed no particular changes, except that the solitary glands were enlarged—especially in its upper portion.

*Anatomic Diagnosis.*—Cholera; acute follicular and necrotic enteritis; follicular colitis; acute parenchymatous nephritis; acute parenchymatous hepatitis; adhesive pleuritis; emphysema of the lungs; persistent thymus.

Tissues from the organs hardened in Zenker's solution.

*Smears.*—Ileum (carbol fuchsin 1:10): Showed almost pure culture of slender rods, often curved, quite numerous; many degenerated, columnar epithelial cells. (See fig. 2.) Spleen (Gram and Safranin): No bacteria. Heart's blood (Gram and Safranin): No bacteria.

*Method of isolation.*—Cultures were made from the ileum according to the Schottelius enriching method. Peptone solutions (1 per cent Witte's peptone and 0.5 per cent NaCl in distilled water) with a reaction of + 1, + 0.5, neutral, and — 1 were inoculated and kept at 35°–37°.

In twenty hours the + 1 tube showed a dense layer of growth near the surface, with beginning pellicle formation. A hanging drop preparation from the surface showed short, actively motile curved rods—apparently in pure culture.

The + 0.5 and neutral tubes were well clouded, no pellicles.

The — 1 tube was faintly clouded with a thin pellicle in the process of formation. It showed actively motile curved rods in the hanging drop.

The addition of ten drops of chemically pure sulphuric acid to each of the tubes gave a distinct indol reaction, which was most marked in the + 1 tube.

Twenty per cent gelatin plates inoculated from the surface growth of the + 1 peptone tube were kept at 18°–28°. In twenty hours pinhead-sized areas of liquefaction were produced. These were cir-

cular, well defined, and contained motile masses of growth of a broken-up, refractile character.

A pure culture was obtained on + 1 agar.

*Biochemical peculiarities and variations.—On + 1 agar* a luxuriant dirty-white growth is seen in twenty-four hours at 35°–37°. Later the edges become crenated—especially if the agar is somewhat dry. The condensation water is densely clouded. In old cultures spine-like processes may project from the edges of the growth, which is much more luxuriant on fresh, moist agar than on the same medium with a slightly dry surface. (For the optimum reaction see under "Liquefaction of gelatin.")

*In + 1 bouillon* the growth is somewhat variable. On one occasion the bouillon may be uniformly clouded; in the following, this is termed the "anaërobic type of growth." Again it may be clouded with a dense layer of growth near the surface, which soon forms a pellicle. Below this is termed the "aërobic type of growth." In a stained preparation from a forty-eight hour culture the organisms are thicker and more curved than from one in peptone solution grown under like conditions. The morphology varies with the reaction of the medium. The character of the growth is independent of the presence or absence of muscle sugar, and is apparently due to the predominance of the aërobic type of the organism on the one hand, or of the anaërobic type on the other. I came to term these aërobic and anaërobic types of growth as a matter of convenience, but a better theoretical explanation is furnished by assuming that the difference is due to a variation in the specific gravity of the bacterial cells. If a number of bouillon tubes be inoculated from an agar slant, and kept under like conditions, some will show the aërobic type while others will be uniformly clouded and may remain so or form a pellicle at a later date. The type of growth can be transmitted by further inoculations in bouillon, although, in the case of the anaërobic type, there is a tendency toward a cropping out of the aërobic one.

*The production of a pellicle in fluid cultures.*—As with the diphtheria bacillus, and other organisms, the habit of producing a pellicle in fluid cultures can be firmly established by transferring a portion thereof through a series of fluid cultures. "579 A" is one, which after being transplanted in this manner at intervals of three or four days for a couple of months, shows little or no ten-

dency to grow in the deeper parts of the bouillon, while a dense layer appears near the surface of the fluid and a pellicle is formed in much less time than when the training process was initiated, and the same result can be obtained much more rapidly by using the pellicle formed on a liquefied gelatin culture. The whole process is, in fact, one of artificial selection. Inoculations from such cultures are usually made from the upper layers of the fluid and hence a series of such inoculations yields an artificially selected race of organisms of low specific gravity.

So far as indol and alkali productions are concerned, there is no difference in the action of the aërobic and anaërobic type of organisms. (See alkali production.)

It is evident that the presence or absence of a pellicle in bouillon cultures is of little value in the differentiation of species.

*Litmus milk* is acidified and coagulated in forty-eight hours. (Control tubes remain sterile.) In about four days a firm clot is formed with separation of the whey and partial reduction of the litmus. The fermentation of lactose bouillon is evidence that this culture produces lactase.

*+ 1 glucose bouillon* is faintly clouded, but the growth occurs mostly at the bottom of the test tube as a stringy, viscous mass. There is no apparent increase after twenty-four hours. (See growth in the fermentation tube.)

*On potato* (*unneutralized*) the growth is variable, sometimes none appearing, or again a slight dirty yellowish one may be seen in three or four days. This variability is probably due to a difference in the acidity of the potatoes used.

*Solidified ox serum* is rapidly digested.

When grown anaërobically (pyrogallic acid method), in + 1 *glucose agar,* growth appears along the line of inoculation, but the culture is no longer viable after the second anaërobic transplanting.

(*a*) ON THE DEMONSTRATION OF THE CHOLERA-RED REACTION.

Immediately after isolation from the body, this organism gave a pronounced cholera-red reaction upon the addition of ten drops of chemically-pure sulphuric acid to cultures grown in peptone solution during eighteen to twenty hours at 35°–37°. Since then, this reaction has only appeared at intervals—even in solutions

prepared from Witte's peptone,[1] which had been set aside as "Proper for Indol."

All the cultures mentioned in this article have shown the same variation from time to time.

For some time peptone solutions of various reactions were used both in isolating the cholera spirillum at autopsy, from stools, water, etc., and in testing for cholera red, but without any constant results which might determine whether any one reaction favors surface growth or the demonstration of the reaction. Dunham's peptone solution containing 1 per cent Witte's peptone and 0.5 per cent sodium chloride in distilled water has a final reaction of +0.5, and has given the best results on the whole. This solution has such poor nutritive qualities for many species of intestinal bacteria that it is especially suitable for isolation by the Schottelius enriching method.

Upon investigating this uncertainty of the cholera-red reaction, I determined to try sugar-free bouillon, which has been shown by Smith [2] to be such an excellent culture fluid for the production of indol by bacteria. In the first batch of this medium, these cultures gave excellent cholera-red reactions, but in two subsequent ones the reaction failed to appear. These three media were shown to be free from nitrites and fermentable sugars, by testing with *B. coli,* as recommended by Smith. In addition to sugar-free bouillon, four different peptone solutions were tested, namely: *Peptone Sicca cum Sale,* R. Nishiyama, Osaka, Japan; *Peptone Carne,* E. Merk, Darmstadt; and two samples of *Peptonum Siccum,* Friedr. Witte, Rostock—one of which had been marked "Proper for Indol." These too were found to be free from fermentable sugars and nitrites, but all failed to yield cholera red. However, they gave the indol reaction upon the addition of a trace of sodium nitrite.

As is well known, the demonstration of the cholera-red reaction depends upon the fact that an organism not only forms indol, but

[1] It may be noted that this so-called "peptone" consists of a mixture of albumoses and contains only a minimal quantity of true peptone—Torald Sollmann, Witte's Peptone: Its Dissociation, and its Combination with Acid and Alkali. *Amer. Jour. of Physiol.,* 1902, VII, 203; on the other hand, there are "peptones" on the market, such as that manufactured by the firm of Chapoteau, which contain as much as 50 per cent of pure peptone (J. P. Pawlow: The Work of the Digestive Glands, 1902, 96).

[2] Theobald Smith: A Modification of the Method for Determining the Production of Indol by Bacteria. *Jour. of Exper. Med.,* 1897, II, 543–547.

also either produces nitrites or reduces nitrates to nitrites. Having a premonition that the inconsistancy of the reaction might depend upon a variation in the amount of nitrates present in different lots of peptone or meat extracts, or upon their accidental introduction on one occasion and not on another (when Cross and Blackwell's table salt is used, much more constant results are obtained, than when C. P. sodium chloride is employed), I prepared peptone solutions in the manner above indicated, but in addition to the C. P. sodium chloride, I introduced 0.01 per cent C. P. sodium nitrate (1 cubic centimeter of a 10 per cent solution per liter). In such a solution the cholera-red reaction is not only constant, but it appears more promptly and is more intense than usual. Control peptone solutions, not containing sodium nitrate, failed to give the reaction. (All the cholera cultures mentioned in this article give the reaction constantly and promptly in this medium.)

Since completing this work, I have found that Max Bleisch,[1] as long ago as 1893, emphasized the necessity of introducing nitrates into the peptone solution.

There seems to be some evidence that the nitrate content of meat extract or "peptone" may vary; and this may account for some of the discrepancies in species description, for an organism attributed with nitrifying powers may only possess the ability to reduce nitrates to nitrites—a property common to many species of bacteria.

Thus, to cite an instance: Last year Woolley and Jobling,[2] working in this laboratory, described cultures of *B. bovisepticus* which gave a distinct cholera-red reaction upon the addition of chemically-pure sulphuric acid to cultures grown for from twenty-four to thirty-six hours in Dunham's peptone solution. At that time the cholera cultures here mentioned also gave the same reaction. I have recently tested two of these cultures of *B. bovisepticus*, and find that they fail to give the cholera-red reaction in the peptone solution in which the cholera cultures fail to do so, but yield it promptly when grown in the peptone solution containing 0.01 per cent sodium nitrate.

As shown by Kastle and Elvone,[3] hyponitrous acid, nitrous acid, nitrites,

[1] Max Bleisch: Ueber einige Fehlerquellen bei Anstellung der Choleraroth reaktion und ihre Vermeidung, *Zeit. für Hyg. und Infekt.*, 1893, XIV, 103–115.

[2] P. G. Woolley and J. W. Jobling: A report on Hemorrhagic Septicemia in Animals in the Philippine Islands, *Bull. No. 9, Biological Laboratory, Bureau of Government Laboratories*, p. 8.

[3] Kastle and Elvove: Oxidation and Reduction in the Animal Organism and the Toxic Action of Powerful Oxidizing and Reducing Substances. *Amer. Chem. Jour.*, 1904, 31, 195–207.

etc., are, in part, converted into nitrates in the animal body. And as has been demonstrated by Mayo,[1] when potassium nitrate is fed to cattle, a chemical test for nitrates can sometimes be obtained after their death, although, as a rule, nitrates are rapidly reduced to nitrites in the tissue fluids.

It seems probable that the use of Smith's sugar-free bouillon, containing 0.01 per cent sodium nitrate, would furnish a means of testing the production of indol and the simultaneous reduction of nitrates by many bacteria.

### (*b*) ON THE LIQUEFACTION OF GELATIN.

At the time of isolation, this organism showed active proteolytic properties—liquefaction of 20 per cent gelatin appearing within twenty-four hours, at 18° to 28°, and rapidly spreading to the sides of the test tube as a shallow, circular, pan-shaped area. Then the liquefaction descended progressively from above downwards, involving the whole width of the tube, with a slight funned-shaped depression in the center along the needle puncture. Careful data concerning the reaction and dryness of the gelatin were not kept at the time, but some variations in the rate and character of liquefaction were noticed. Further work at the time being impossible, the original agar culture was kept in the ice chest (transplants on +1 agar being made at intervals of every two months) for eight months. The organism still showed the above type of liquefaction (which is often described as being characteristic of Spirillum Finkler Prior) at 18°–28° in 20 per cent gelatin, which had a final reaction of +1.2. At the same temperature, in fresh 20-per cent gelatin, which had a final reaction of +2, the organism slowly produced, in the course of three days, a small turnip-shaped area of liquefaction which, drying at the surface, left a small bubble-like depression—that is, it produced the type of liquefaction which was described by Koch as being characteristic of the cholera spirillum.

In two separate trials with the same gelatin (+2) at 35°–37°, the inoculated material precipitated, and no growth or liquefaction occurred.

In +1.5 gelatin at 10°–15° no growth occurred in ten days, but rapid liquefaction took place on change to 18°–25°.

When grown anaërobically (pyrogallic acid method) at 18°–28°

[1] N. S. Mayo: Cattle Poisoning by Nitrate of Potash, *Bull. No. 49* (1895), Kansas State Agricultural College.

in +1 gelatin containing muscle sugar, growth appears along the stab but no liquefaction takes place in three days.

Before detailing some experiments performed to determine the factors influencing variation in the type of liquefaction, it may be well to note some of the points brought out in the literature on this subject.

The proteolytic ferments of bacteria are only active in a medium alkaline to litmus, and it takes but a small amount of acid to hinder their action. This is in accord with the behavior of trypsin. When carbohydrates which can be so fermented as to form acids are present in gelatin, its liquefaction is inhibited. In 1898 Auerbach,[1] working with a number of liquefying bacteria, showed that the inhibiting power of glucose exceeded that of lactose, and that in the case of *B. vulgare* the acid products of fermentation inhibited the formation of the ferment itself. It seems that for the production of the ferment a medium containing albumin and the access of free oxygen is necessary. According to Liborius[2] liquefaction of gelatin takes place very slowly in the absence of oxygen—with the exception of the case of some anaërobes.

According to T. Sollman (loc. cit, p. 211), "Kühne investigated the action of *B. subtilis* and *B. prodigiosus* on solutions of protalbumose from the chemical standpoint, and found that the phenomena resemble closely those of tryptic digestion. The conversion to tyrosin, leucin, and tryptophan was often almost complete." Again, according to Gotschlich,[3] "Kalischer in experiments to determine how much of the casein splitting was due to the ferment and how much to the living cells, found that the ferment was able to produce peptone, leucin, tyrosin, as well as ammonia and aromatic oxyacids—in which its action also is in harmony with that of trypsin."

The melting point of gelatin undoubtedly plays a part in influencing the type of liquefaction which will occur at any given temperature. In my own experience the addition of alkali lowers the melting point. Thus, neutral gelatin which will not congeal at 18°–28° will do so in the ice chest, and + 1 gelatin is not as solid as + 1.5 gelatin at the same temperature. An interesting communication by Paul von Schroeder[4] throws some light on this subject. "When a gelatin solution is heated at 100°, and samples are taken out at intervals and placed in a thermostat at 25°,

[1] Auerbach: Ueber die Ursache der Hammung der Gelatinverflüssigung durch Bakterien durch Zuckerzusatz. *Ref. C. B.*, II *Abt.* 1898, IV, 492–494.

[2] Liborius: Beitrage Zur Kenntniss des Sauerstoffbedürfnisses der Bacterien, *Ztschr. f. Hyg.*, 1886, I, 115–176.

[3] E. Gotschlich: Handbuch der Pathogenen Mikroorganismen, Kolle u. Wassermann, 1903, I, 107.

[4] Paul von Schroeder: Phenomena of the Setting and Swelling of Gelatin, Review: *Jour. of the Chem. Soc'y*, 1903, vol. 84, ii, 721.

their viscosity being determined five minutes later, it is found that the values of the viscosity diminish, as the duration of the heating at 100° increases, ultimately becoming constant.

"This change is attributed to a process of hydrolysis * * *.

"Certain salts increase the viscosity, magnesium salts exerting the greatest influence * * *.

"The effect of hydrochloric acid and sodium hydroxide on the behavior of gelatin solutions was similarly studied. The process of hydrolysis is accelerated by both hydrogen and hydroxyl ions, and the final value of the viscosity thus attained after hydrolysis is lower than that reached in pure or salt containing gelatin solutions."

Again, according to Rousseau,[1] if gelatin be dialized, so as to remove the calcium salts contained therein, one obtains a solution which, sterilized in an autoclave at 120° for twenty to thirty minutes, solidifies upon cooling.

In order to determine what influence the reaction and dryness of the gelatin exert upon the type of liquefaction produced by a given cholera culture, the following experiment was performed: Nutrient gelatin was prepared containing 20 per cent gold label gelatin, 1 per cent Witte's peptone and 0.3 per cent Liebig's beef extract. It was divided into halves and to each portion normal NaOH was added, one-half receiving more than the other. After sterilization one portion showed a final reaction of +0.8, while that of the other was +1.0. In addition to this, another sample of gelatin, slightly darker in color but prepared in the same way, which had been kept on ice for three weeks and showed some evaporation and a final reaction of +1.5, was used. This one was melted and resolidified before inoculation. Each sample contained a small amount of muscle sugar as shown by subsequent fermentation with *B. coli*.

Four tubes from each of these samples were then inoculated from a twenty-four-hour culture of "579" on +1 agar, which had been kept on agar transplants at 35°–37° for two and a half months. In forty-eight hours at 18°–28° there was quite a noticeable variation in the amount of liquefaction produced in the different sets of tubes. The amount of this, in the four tubes of any one of the three sets, was not exactly uniform, probably on account of a variation in the number of bacteria introduced at the time of inoculation,

[1] Em. Rousseau: Influence of the Salts of Calcium upon the Solidification of Gelatin Sterilized at 120°. *Bull. Inst. Pasteur*, 1903, I, 719.

but the difference between the three sets was very noticeable. Any one of the four +0.8 tubes showed more advanced liquefaction than any of the +1 tubes, and the difference between the +1 and +1.5 tubes was still more marked, as shown in fig. 1.

It is often stated that "bacterial proteolytic enzymes, like trypsin, show increased activity in the presence of certain chemicals, such as sodium carbonate and salicylate."

So far as the action of certain ions upon the tryptic digestion of fibrin is concerned, A. Kanitz,[1] in reviewing the work of Dietz and confirming the quantitative experiments of Shields,[2] has shown that the optimum concentration of the hydroxyls from barium, strontium, and calcium hydroxides varies between $\frac{1}{70}$ and $\frac{1}{150}$ of the gram-molecule per liter. Determination of the electric conductivity and other physical constants shows that these alkaline earths are strongly and almost equally dissociated at these dilutions, and since the three hydroxides work at the same concentration he concludes that the kation is without influence and that the anion is alone active. He then calculated, from the per cent of hydrolysis of potassium carbonate in given dilutions, the concentration at which the carbonate of potassium exerted the most active influence on tryptic digestion and found this to be about $\frac{1}{200}$ of the gram-molecule per liter. He was unable to say that there was any difference in the mode of action of carbonate of potassium and the hydroxides of the alkaline earths. Kanitz concludes that the optimum for tryptic digestion is a liquid containing $\frac{1}{70}$ to $\frac{1}{200}$ of the hydroxyl ion (OH=17 gms.) per liter.

In order to test the above statement from a bacteriological standpoint, an experiment was performed as follows: I prepared one liter of nutrient gelatin by adding 20 per cent Gold Label gelatin, 1 per cent Witte's peptone, and 0.5 per cent sodium chloride to 1,000 cubic centimeters of distilled water; the ingredients were then dissolved by boiling; distilled water added to 1,000 cubic centimeters; the mixture then was divided into two parts of exactly 500 cubic centimeters each; each half titrated to phenolphthalein, and sufficient normal NaOH added to one-half to give a reaction of +1,

[1] A. Kanitz: Ueber den Einfluss der Hydroxylionen auf die tryptische Verdauung. *Zeit. für physiol. Chemie.*, 1902, 37, 75–80.

[2] John Shields: Ueber Hydrolyse in wässerigen Salzlösungen. *Zeit. f. physikalische Chemie.*, 1893, 12, 167.

and an equal volume of normal $Na_2Co_3$ was then added to the other half; after cooling to 40° the whites of three eggs were added to each half; each portion was then boiled for three minutes, filtered through cotton, distributed and sterilized in the Arnold for twenty minutes on each of three successive days. The final reaction of the NaOH gelatin was +1.7, while that of the $Na_2Co_3$ gelatin was +1.8.

Six tubes of each were then inoculated from the same place on the edge of the growth of a twenty-four-hour agar culture of "579," and kept at 18°–28°. Liquefaction commenced and progressed slowly but equally during several days' observation.

The results of Kanitz would not appear to give conclusive proof of his view that the Kation exerts no influence on the optimum reaction, as a glance at the table given in his paper will show, the variations between the three alkaline earths being quite marked at different temperatures. In Dr. Wherry's work he compared equivalent amounts of soldium hydroxide and sodium carbonate, and thus, while he had the same concentration of hydroxyl ions, he had, in the case of the latter reagent, twenty-five times the number of sodions present in unit volume. This latter fact would tend to show that the kation is without marked influence; at least in the case of sodions. However, these results show that the question is one which is barely touched and is well worthy of complete investigation by a use of the methods of physical chemistry in biology.—Freer.

An attempt was made to increase the proteolytic activity of this culture by transferring it at intervals of every few days from one gelatin tube to another. At the end of four months this culture showed no greater activity than another transplant of the same culture which had been kept on agar slants for the same length of time.

What has already been said concerning the influence of the reaction of the medium upon the rapidity with which the cholera spirillum is able to liquefy gelatin has a direct bearing upon the type of liquified areas it will produce in or upon gelatin plates. Further, when, as has been noted by many observers, the same culture gives rise to two distinct types of liquified areas, the difference may be explained by the relation of the plated organisms to their oxygen supply. Thus, an organism situated at the surface on account of its greater supply of free oxygen might be expected to produce a more rapidly spreading area of liquefaction than one more deeply situated, where the supply is *relatively* less. Moreover,

the colony at the surface would be of the shallow, turbid type with a greater area than that of the deeper colony where the organisms encounter a greater resistance of the surrounding gelatin, and in consequence of which they would be massed together—producing the refractile "ground-glass" type of colony. It has been noted that such "ground-glass" colonies, upon further growth, invariably break up into liquid areas with turbid contents and such breaking up occurs *pari passu* with a lessening of the surrounding resistance and an increase in the supply of free oxygen. That such a supply of free oxygen does exist in a thin layer of gelatin can be proven by covering the opening of a gelatin stab culture with a few drops of liquid gelatin. Here no liquefaction occurs until the organisms have spread nearly to the surface.

### (*c*) ON THE OPTIMUM REACTION.

All the cultures mentioned in this article show much more luxuriant growth in eighteen to twenty hours, at 35°–37°, on fresh —0.5 than on fresh +0.5 agar. Furthermore, the maximum amount of growth is obtained on —0.5 agar in eighteen to twenty hours, while that on +0.5 agar does not equal it in thirty-six to forty-eight hours at the same temperature. The —0.5 agar was prepared from Liebig's beef extract and had an initial acidity of 1 per cent acid to phenolphthalein. It was neutralized and brought to a reaction of —1. After sterilization, the reaction was reduced to —0.5. Since 20 cubic centimeters of normal NaOH were added in the first place, and part of this was lost in the precipitate thrown down by autoclaving, it contained between 1/50 and 1/100 of a gram-molecule per liter. This would seem to support the idea that the optimum conditions for the growth and multiplication of the cholera spirillum are such as will favor its proteolytic activity.

### (*d*) ON THE PRODUCTION OF ALKALI.

Fifty cubic centimeters of Smith's sugar-free bouillon[1] was placed in each of five 600-cubic centimeter Ehrlenmeyer flasks and autoclaved at 120°. Final reaction, +1.5.

Each was then inoculated with one loop from an eighteen-hour

[1] For the method of preparation see *Jour. of Exper. Med.*, 1899, IV, 375.

evenly clouded sugar-free bouillon culture of "579," and the following table illustrates the rate of alkali production:

| No. of flask. | Temperature. | Age of culture. | Reaction to phenolphthalein. | Remarks. |
|---|---|---|---|---|
| | ° | *Hours.* | | |
| 1 | 30–35 | 24 | +0.8 | Dense cloudiness; no pellicle. |
| 2 | 30–35 | 48 | +1.0 | Dense cloudiness; no pellicle; actively motile curved rods. |
| 3 | 30–35 | 72 | +0.2 | Do. |
| 4 | 30–35 | 96 | Neutral. | Do. |
| 5 | 30–35 | 120 | −0.8 | Dense cloudiness; no pellicle; rods not so actively motile; few curved filaments. |

Alkali production progresses equally as well when a pellicle is formed. It also occurred in unneutralized sugar-free bouillon with an initial acidity of +2.3. If the sodium chloride usually added to sugar-free bouillon be left out, no formation of alkali can be detected by titration with phenolphthalein—at least during five days. In control flasks of the same bouillon, plus sodium chloride, alkali production occurred about as rapidly as shown in the above table. This would seem to indicate that the alkali is produced by a conversion of NaCl into NaOH or $Na_2Co_3$, and that the greater part of the alkalinity is owing to the formation of such substances rather than to ammonia, amine, and ammonium bases, to which it is usually attributed. However, it is also possible that sodium chloride exerts a catalytic (accelerating) action on the formation of ammonia.

## III. A DESCRIPTION OF THE SOURCE AND ISOLATION OF FIVE OTHER CHOLERA CULTURES, AND OF THEIR RESEMBLANCE TO ONE ANOTHER AND TO CULTURE "579.

*Cholera "Scout"* is a culture obtained by the Schottelius enriching method from a stool sent to the laboratory from Caloocan on April 16, 1903. The patient was a native scout who died on the next day with typical symptons of Asiatic cholera, which was epidemic in Caloocan and the surrounding country at the time. In its cultural characteristics it is indistinguishable from "579," excepting that litmus milk is acidified in twenty-four hours at 37°, but no coagulation occurs in five days. Hence, like "579," it

produces lactase, and differs from it in the absence of the production of rennin.

*Cholera "561"* is a culture obtained by the Schottelius enriching method from Eugenia Holandes, a Filipina, 33 years old, who died on March 26, 1903, after an illness of three days. Autopsy, seven hours after death. To briefly summarize the post-mortem findings: The skin of the hands and feet is shrivelled; the feet are in extreme flexion with the toes in extension; the pleural and peritoneal membranes covered with a sticky secretion. Cloudy swelling of the solid organs. Ileum congested, especially in its lower half, the mucosa showing some patches of epithelial desquamation. Contents of ileum greenish black and containing much mucus. Old and advanced amebic colitis. Culturally it is indistinguishable from Cholera "Scout."

*Cholera "A"* is a culture obtained at autopsy by Dr. R. P. Strong some time in the fall of 1903, cholera being endemic in Manila at the time. Culturally it is indistinguishable from cholera "Scout."

*Cholera "City Moat"* is a culture obtained by Mr. Lindquist, of the First Reserve Hospital laboratory, from the city moat near the hospital, about July, 1903. Cholera was endemic in Manila at the time. Culturally it is indistinguishable from cholera "Scout."

*Cholera "Pfeiffer"* is a culture of that name brought by Dr. R. P. Strong from Germany. It has been grown on artificial media for a period of nine years, and during the past year has not been passed through animals. Culturally it is indistinguishable from cholera "Scout," but it is very much more sensitive to the action of agglutinating sera.

*"554–B"* is a culture obtained on March 20, 1903, from a cholera autopsy. Morphologically it appears as short, curved, actively motile rods. It closely resembles the above cultures but does not agglutinate with the serum of a rabbit immunized against "579" nor with the serum of a cholera convalescent.

## IV. THEIR GROWTH IN THE FERMENTATION TUBE IN THE PRESENCE OF CARBOHYDRATES.

Medium: Smith's sugar-free bouillon, which had a final reaction of +1.5 containing 1 per cent of glucose, maltose, saccharose, and lactose. One per cent of starch was added to some of the same bouillon and autoclaved after distribution; the initial reaction was

not changed. When inoculated with "579" and kept at 35°–37° the following results were noted:

| Bouillon. | Gas. | Reaction of contents of bulb and neck on fourth day. | Gas in control tubes inoculated with *B. coli* (fourth day). | Remarks. |
|---|---|---|---|---|
| Glucose | 0 | +3.3 | 30 per cent; $\frac{H}{CO_2}=\frac{2.5}{1}$ | Maximum growth attained in twenty-four hours. Bulb and closed branch turbid; no pellicle. Agar slants inoculated after twenty-four hours from the bulb or neck remain sterile. |
| Maltose | 0 | +3.2 | 45 per cent; $\frac{H}{CO_2}=\frac{3}{1}$ | Maximum growth attained in twenty-four hours. Bulb and closed branch turbid; no pellicle. Agar slants inoculated on the fourth day from the bulb or neck remain sterile. |
| Saccharose | 0 | +3.5 | No gas; closed arm cloudy. | Do. |
| Lactose | 0 | +4.0 | 40 per cent; $\frac{H}{CO_2}=\frac{2}{1}$ | Maximum growth attained in twenty-four hours. Bulb and neck turbid, *closed branch clear;* no pellicle. Growth more dense than in other sugars. Agar slants inoculated on the fourth day from bulb or neck show a luxuriant growth in twenty-four hours at 37°; actively motile curved rods in the hanging drop. |
| Starch[1] | 0 | +4.0 | No gas; closed arm cloudy. | Maximum growth attained after twenty-four hours. Culture viable on fourth day as per lactose tube. |

[1] A test tube containing the same starch solution became densely turbid and a well-marked pellicle was formed. On the fourth day the acidity had reached 3 per cent The closed arm of a fermentation tube was filled with this culture and — 1 bouillon added. When inoculated with *B. coli* 30 per cent of gas was formed in forty-eight hours $\frac{H}{CO_2}=\frac{3}{1}$.

It will be noticed that in glucose, maltose, and saccharose bouillon there was growth in the closed arm as well as in the bulb, and that the acids produced were of such a character as to destroy the vitality

of the organism. On the other hand, in the case of the lactose and starch bouillon, no growth occurred in the closed arm, and, although a greater quantity of acid was produced, the organism was still viable on the fourth day.

In another series of experiments, in which 0.5–1 per cent glucose bouillon (final reaction = +1.5) was distributed in small flasks and inoculated from the same culture and kept at 35°–37°, the maximum amount of acid (3–3.5 per cent) was produced in twenty-four hours and transplants made at that time remained sterile.[1]

Again, enough normal NaOH was added to sugar-free bouillon to give a calculated neutral reaction. The final reaction after autoclaving was +0.7. A sterile solution of glucose, amounting to $\frac{1}{4}\frac{1}{0}$ per cent, was then added and the flask inoculated and kept at 28°. In four days the acidity had been raised 0.5 per cent and the culture was still viable. The experiment was not carried on for a sufficient length of time to note whether the acid produced would be finally neutralized by such alkali production as normally takes place in sugar-free bouillon, but this is hardly probable as the growth, in the presence of even such a small per cent of glucose, is rapidly precipitated and forms a very viscous sediment.

The other cultures grown in solutions of these carbohydrates (reaction +1– +1.5) yielded similar results as shown in the following table:

| Bouillon. | "Scout." | "561." | "A." | "City moat." | "Pfeiffer." | Remarks. |
|---|---|---|---|---|---|---|
| Glucose | +3.8 | +3.6 | +3.8 | +3.5 | +3.5 | Titration on fourth day. Character of growth and fate of culture as per culture "579." |
| Maltose | +4.0 | +4.0 | +3.0 | +4.0 | +4.4 | Do. |
| Saccharose | +3.0 | +3.0 | +3.0 | +3.0 | +3.0 | Do. |
| Lactose | +3.8 | +4.0 | +4.5 | +4.3 | +4.0 | Do. |
| Starch | +2.8 | +3.0 | +3.0 | +2.3 | +2.8 | Do. |

Buxton,[2] in an excellent discussion on bacterial enzymes states that "cholera then produces amylase, maltase, but no invertase,

[1] See analogy in the case of the diphtheria bacillus (Th. Smith, loc. cit., p. 382). It is extremely probable that any toxin formed by the cholera spirillum would be destroyed in a manner similar to that which takes place in diphtheria cultures.

[2] Buxton: Mycotic Enzymes. *Am. Med.*, July 25, 1903, 138.

lactase, nor inulase." These cultures seem to produce both lactase and invertase. The sugars used were prepared by Merk.

E. Gotschlich (loc. cit., p. 106) states that Fermi and Montesano found that invertin occurred inconstantly in the cholera spirillum and spirillum of Metchnikoff.

## V. THEIR RELATIONSHIP, AS SHOWN BY AGGLUTINATING AND BACTERICIDAL SERA.

In applying the Gruber-Durham test to the study of the identity or relationship of the following cultures, a number of facts observed by others influenced both the choice of the method employed and the interpretation of the results.

In making a quantitative determination of the power of a given serum to produce a complete agglutinate when tested on a series of cultures, probably no one factor will influence the production of discordant results so much as quantitative variations between the agglutinin and the agglutinable substance. Thus, to cite an instance, an emulsion of culture "579" in 0.8 per cent sodium chloride solution was tested against the serum of a cholera victim diluted 1:100; agglutination was partial in thirty minutes and not complete until sixty minutes at 28°. The same emulsion was diluted with an equal quantity of the salt solution and then at 1:100 gave a complete reaction in thirty minutes at the same temperature.

A dense suspension of a culture when mixed with a powerful serum at a low dilution may give a prompt but only a partial reaction—numerous bacilli remaining unaffected in the serum which is now freed from agglutinins by the precipitated bacteria. On the other hand, as the dilution of the serum is increased, a similar disproportion is produced with the same result.

On account of variations in the density of the growth in bouillon, which the cultures studied at times show, emulsions of the bacilli in 0.8 per cent sodium chloride solution were exclusively employed. The cultures were grown on +1 agar for eighteen to twenty hours at 35°–37°, and the emulsions made to correspond as nearly as possible with the density of a twenty-four-hour typhoid culture according to the method employed by Smith [1] in the comparative

[1] Theobald Smith: *Jour. of Exper. Med.*, 1898, III, p. 465.

study of tubercle bacilli. They were allowed to stand for ten minutes in order to give time for the coarser particles to settle. Such an emulsion is microscopically free from clumps, and the rods retain their active motility in the control drops for an hour or more.

The serum was diluted with 0.8 per cent sodium chloride solution in Thoma-Zeiss blood pipettes, and a loopful of this serum was then mixed with an equal quantity of the emulsion and examined from time to time with the 1/6 objective. It will be noted that the dilution of the serum in the drop was always twice that in the diluting pipette. Control hanging drops of the emulsion were always made and examined before and at the close of each experiment. The microscopic method was employed because it was believed that the end of the reaction can be more accurately determined and any differences in the character of the clumps noted.

It is a well-known fact that organisms, which have been grown for a long time upon artificial media, are more sensitive to the action of homologous sera than they are when their pathogenicity has been raised. Typhoid cultures recently isolated from the body sometimes show a marked resistance to agglutination with the patient's serum as compared with old laboratory cultures. As shown by F. Hamburger [1] the agglutinability of cholera cultures diminishes with an increase in virulence.

AGGLUTINATION WITH THE SERUM OF A CHOLERA VICTIM.

The history of the serum is briefly as follows: Candido Nugin, a Filipino, 19 years old, died at the San Lazaro Cholera Hospital on January 8, 1904. He was ill for thirteen days; had rice-water stools during the acute stage of the disease, passed into the typhoid stage, and died on the thirteenth day with symptoms of acute nephritis. At the autopsy six hours after death, the kidneys showed acute parenchymatous nephritis; there was cloudy swelling of the liver and heart muscle. The ileum was still in a congested state, but its mucosa was in fairly good condition. Smears from the ileum showed a number of thin curved rods, mixed with many other organisms. No cultures were made.

The following table shows the agglutinating action of the serum from the hearts blood of this patient in such dilutions as were tested.

[1] F. Hamburger: *Wien. Klin. Woch.*, 1903, XVI, 97–98.

An accident to the serum prevented the determination of its agglutinating limit:

| Culture. | Temperature. | Dilution of serum. | Result. |
|---|---|---|---|
| | ° | | |
| "579" | 28 | $\frac{1}{100}$ | Small motile clumps in seventeen minutes; complete in thirty minutes. |
| "579" | 28 | $\frac{1}{200}$ | Small motile clumps in ten minutes; complete in forty minutes. |
| "Scout" | 28 | $\frac{1}{100}$ | Almost complete in thirty minutes; complete in sixty minutes. |
| "561" | 28 | $\frac{1}{100}$ | Do. |
| "A" | 28 | $\frac{1}{100}$ | Almost complete in thirty minutes; not complete in sixty minutes. |
| "City moat" | 28 | $\frac{1}{100}$ | Complete in thirty minutes. |
| "Pfeiffer" | 28 | $\frac{1}{100}$ | Complete in twenty minutes. |
| "554b" | 28 | $\frac{1}{40}$ | Negative during an hour's observation. |

My own serum diluted 1:20 produced no agglutination during forty-five minutes' observation.

In this experiment no attempt was made to use salt-solution suspensions of equal density and the variation in the time when complete agglutination occurred is noticeable.

AGGLUTINATION WITH IMMUNE RABBIT SERUM.

A rabbit was injected with 0.8 per cent sodium chloride suspensions of culture "579" grown on +1 agar for twenty-four hours at 35°–37°. It received the contents of about six agar slants subcutaneously and intraperitoneally during two months. In this twenty-four-hour-old serum the agglutinating limit is not great, but is considered sufficient for the following comparative and quantitative estimations:

| Culture. | Temperature. | Dilution of serum. | Result. | Remarks. |
|---|---|---|---|---|
| | ° | | | |
| "579" | 25 | $\frac{1}{800}$ | + | Complete in thirty-five minutes; small, compact clumps.[1] |
| "579" | 25 | $\frac{1}{2000}$ | — | Partial in twenty-five minutes; not complete in sixty minutes.[1] |
| "Scout" | 28 | $\frac{1}{800}$ | + | Complete in thirty-five minutes; small, loose clumps.[1] |
| "561" | 28 | $\frac{1}{800}$ | + | Do.[1] |

[1] The hanging drop was not examined during the five minutes previous to the given time, hence it is probable that the table indicates a greater uniformity in this respect than occurred in reality. (See footnote under "Morphology and pleomorphism.")

| Culture. | Temperature. | Dilution of serum. | Result. | Remarks. |
|---|---|---|---|---|
| "A" | 28 | $\frac{1}{800}$ | + | Complete in thirty-five minutes; small, compact clumps.[1] |
| "City moat" | 28 | $\frac{1}{800}$ | + | Complete in thirty minutes.[1] |
| "Pfeiffer" | 28 | $\frac{1}{800}$ | + | Complete in thirty-five minutes; small, loose clumps.[1] |
| "554b" | 28 | $\frac{1}{200}$ | — | Negative in thirty-five minutes. |

[1] The hanging drop was not examined during the five minutes previous to the given time, hence it is probable that the table indicates a greater uniformity in this respect than occurred in reality. (See footnote under "Morphology and pleomorphism.")

The normal blood of a control rabbit gave no agglutination at 1 : 10 in forty-five minutes at 28°.

All of these cultures have been grown upon artificial media for from six to twelve months, with the exception of " Pfeiffer," which, as already stated, has been grown on artificial media for the past nine years. Cholera "Pfeiffer" agglutinates almost immediately at a 1 : 40 dilution, whereas it takes several minutes to produce complete results with the other cultures at this dilution. This susceptibility is not noticeable at the higher dilutions.

### PFEIFFER'S REACTION (PERFORMED IN VITRO AFTER THE METHOD OF BORDET).

A loopful of the sodium chloride suspension of the culture to be tested was mixed with a loopful of the above-mentioned immune rabbit serum; a loopful of this mixture then added to a loopful of normal rabbit serum and the result watched in the hanging drop. All of the cultures, with the exception of "554b," agglutinated, the rods became swollen and globular, and in about three hours at 28° began to break up into granular masses; "554b" agglutinated, the rods became swollen but did not disintegrate. In control drops of immune serum alone the rods agglutinated, but no bacteriolysis occurred. In control drops of normal serum the rods retained their motility for three hours.

## VI. THEIR PATHOGENICITY.

### GUINEA PIGS.

In order to save guinea pigs for other purposes, only the pathogenicity of culture "579" toward these animals has been tested. At the time of isolation about 2 cubic centimeters of a twenty-four-

hour bouillon culture injected intraperitoneally killed a fair-sized guinea pig within twenty-four hours. Eleven months later, after direct passage through three guinea pigs, one loop [1] of a twenty-four-hour +1 agar culture, grown at 35°–37°, killed a 482-gram guinea pig in four hours. The peritoneal and thoracic cavities showed intense congestion with sero-sanguineous extravasations. The small intestine was greatly congested (much more so than the large) and filled with a yellowish mucoid fluid containing many desquamated epithelial cells, but, microscopically, no cholera spirilla. The abdominal organs were bound together by a fibrinous exudate. Pure cultures were obtained from the peritoneal cavity on agar plates. There were no organisms in the heart's blood.

PIGEONS (FULL GROWN AND OF ABOUT THE SAME SIZE).

Cultures " 579," " Scout," "City moat," and "561" were pathogenic when one loop of a twenty-four-hour —1 agar culture suspended in salt solution was injected deep into the pectoral muscle. One loop of culture "A" failed to kill a pigeon. Five loops (about 30 milligrams) of culture "Pfeiffer" failed to kill. Abstracts of the protocols are as follows:

*Pigeon 1.*—One loop of "579" deep in left pectoral muscle. Dead in fifty-four hours. Congestion of cutaneous and deep vessels of left side. Cloudy swelling of left pectoral. Intestines congested. Microscopically, many curved rods in left pectoral, none in heart's blood. Many Halteridia and shadow corpuscles in blood. Pure cultures were obtained from the left pectoral muscle and heart's blood (three colonies per loop), which agglutinated with the "579" immune rabbit serum at 1:200 in about twenty minutes.

*Pigeon 2.*—One loop of "Scout" deep in left pectoral; dead in twenty hours; tissue changes as in Pigeon 1; organisms present in pectoral and heart's blood microscopically; many Halteridia present; pure cultures from pectoral and heart's blood which agglutinated with "579" rabbit serum at 1:200.

*Pigeon 3.*—One loop of "City moat" deep in left pectoral; dead in thirty-four hours; tissue changes similar to first case; organisms numerous at seat of injection, not found microscopically in heart's blood, which contained numerous Halteridia and many shadow corpuscles. Pure cul-

[1] The same loop was used throughout the following experiments. When it holds just sufficient culture to fill the cavity of the loop and form a rounded surface on each side, its contents weigh 7 milligrams (wet). Allowing 1 milligram for loss during manipulation "one loop" signifies about 6 milligrams of the culture.

tures obtained from pectoral and heart's blood, which agglutinated with "579" rabbit serum at 1:200.

*Pigeon 4.*—One loop of "561" deep in left pectoral; dead in forty-four hours; tissue changes as above; curved rods at site of injection and quite a number in the heart's blood; few Halteridia; cultures from pectoral and heart's blood pure and agglutinate with "579" rabbit serum at 1:200.

*Pigeon 5.*—One loop of "A" deep in left pectoral; alive and well on tenth day; blood from foot shows very few Halteridia.

*Pigeon 6.*—One loop of "Pfeiffer" deep in left pectoral; alive and well on tenth day. No Halteridia found in blood from foot.

*Pigeon 7.*—Two loops of "Pfeiffer" deep in left pectoral; alive and well on sixth day. No Halteridia found in blood from foot.

*Pigeon 8.*—Five loops of "Pfeiffer" deep in left pectoral; alive and well on fourth day; blood from foot shows a number of Halteridia.

The dose injected into these pigeons seems to be rather large, but was adopted on account of the age of the cultures. I have not been able to test the relative resistance of a pigeon showing marked Halteridium infection on the one hand and one free from it on the other hand, on account of the difficulty of obtaining uninfected pigeons. Culture "Pfeiffer" has been grown on artificial media for such a great length of time that it could hardly be expected to be pathogenic except in large doses. (See footnote under "Morphology and pleomorphism.")

*Monkeys.*—Several attempts to infect monkeys by feeding have been performed with negative results, but I have notes on one case only.

An old adult male monkey (*Macacus cynamolgus*) received the contents of a recent agar slant culture of "579" suspended in — 1 bouillon. This was injected by means of a catheter into the stomach. He remained perfectly well for twenty-four hours. During the next four days 35 cubic centimeters of native spirits, called "arac" (containing about 40 per cent alcohol), were injected into his stomach. During part of the time he appeared to be intoxicated and refused to eat. Five cubic centimeters of 1 per cent sodium carbonate was injected into his stomach, followed by the contents of three + 1 agar slants of "579" suspended in — 1 bouillon. He did not vomit; faeces were normal, and he remained well during a week's observation. The culture "579" had been grown on artificial media for nine months without passage through an animal.

## VII. THEIR MORPHOLOGY AND PLEOMORPHISM.

Each of these cultures shows that tendency toward pleomorphism, which is quite as marked as that seen in cultures of *B. pestis* or *B. mallei*, and which is so confusing to the beginner.

It is generally admitted that no two separate lots of media, which are identical in composition and reaction, can be prepared; and though the methods recommended by the American Committee, and the somewhat modified ones employed in these experiments give

comparable results so far as the reaction may indicate uniformity in composition, some bacteria will point out variations not detectable in other ways. I have not been able to reproduce exactly the same type of morphology in any two successive cultivations of the same culture, even on agar from the same batch, although precautions were taken to grow the cultures under apparently identical conditions, to make the preparations from corresponding portions of the growth, and to subject them to, as nearly as could be judged, like conditions of heating, staining, etc. On agar made on separate occasions the variation is still more marked.

In the case of the cholera spirillum which reaches its maximum growth on moist agar in such a short time, variations in morphology will be shown in preparations from the same culture. Thus, one made from the *edge* of a streak on an agar slant, where the younger forms are still multiplying, may present an entirely different appearance from one made from the *center* of the streak, where the older forms have lengthened out and are undergoing involution changes, as shown in figs. 7 and 8.

The variation which is so striking in this instance is not always so apparent in any of the cultures under consideration, nor is it appreciable in a twenty-four-hour culture of the less pleomorphic *B. coli.* Still this difference in the morphology of the younger and older forms of the cholera spirillum must be taken into consideration in comparing the morphology of different cultures, and when it is taken into account the variation on agar from the same batch may not be so marked.[1] (Compare figs. 8, 9, 10, 11, 12.)

It is hardly necessary to say that no permanent variations in morphology were produced in any culture. Figure 5 represents one which was kept in bouillon for ten months. This culture has been described under "Pellicle production in fluid cultures" as "579A." It will be noted that the organisms are somewhat larger than those pictured in figs. 2, 3, and 4. This is but a temporary modification, transmitted while the culture is kept in bouillon, but

[1] It seems worthy of note that in the preparation of the saline emulsions, for the agglutination and animal inoculation experiments heretofore cited, no such precautions were taken. It seems that the failure to do so may account for some of the variations observed. It is not at all impossible, for example, that a "loop" of young healthy cholera spirilla, taken from the edge of the growth on an agar slant, would exert a greater pathogenic action than one taken from the center where the growth is composed of old semidegenerate individuals.

very soon reverting to the shorter, thinner type when grown in peptone solution or transplanted upon +1 agar.

Figure 6 shows some of the long straight and spiral threads which grow out in the pellicle which forms on a liquefied gelatin culture. These are undoubtedly involution forms, for when a portion of such a pellicle is transplanted upon +1 agar, the short curved forms ordinarily met with upon agar develop abundantly. Further, in stained preparations from such an agar surface these threads and spirals take the stain poorly, and after two or three transplants disappear entirely.

## VIII. SUMMARY AND CONCLUSION.

(1) The substance of this article consists, essentially, in making a careful preliminary study of the variations which occur in one culture of the cholera spirillum, and then comparing it with cultures from different sources.

(2) Certain reasons are given for adopting a modification of the methods of neutralizing media recommended by the American Committee—the hydrogen ion being left out on account of its toxic action.

(3) The cholera spirillum is not a nitrifying organism, and the successful demonstration of the "cholera-red reaction" in a solution of Witte's "peptone" depends upon the presence of a trace of nitrates. Certain reasons are given for presuming that a variation in the nitrate content of media exists.

(4) The type of liquefaction produced in gelatin is influenced to a marked degree by the reaction and melting point of the gelatin. Sodium carbonate does not exert a more favorable influence on the proteolytic activity of the cholera spirillum than sodium hydroxide—at least so far as the liquefaction of gelatin is concerned. The proteolytic activity of a culture could not be increased by passage through a series of gelatin tubes.

(3) The optimum condition for growth is furnished by an albuminous medium containing between 1/50 and 1/100 of a gram-molecule of NaOH or $Na_2CO_3$ per liter, and this corresponds fairly well with the optimum conditions for the tryptic digestion of fibrin.

(6) Alkali, detectable by titration with phenolphthalein, is not produced in sugar-free bouillon devoid of sodium chloride.

(7) Growth in the presence of carbohydrates reveals that the acids produced from glucose, maltose, and saccharose rapidly kill

the cholera spirillum, while those produced from lactose and starch are not toxic—at least within a given time.

(8) The cultures studied are specifically the same as shown by the Gruber-Durham and Pfeiffer reactions. In order to obtain comparable results, quantitative variations between the agglutinin and agglutinable substance were excluded as far as possible.

(9) The pathogenicity for guinea pigs, pigeons, and monkeys is mentioned.

(10) Upon comparing the morphology of the different cultures it was noted that if precautions be taken to make preparations from corresponding portions of the growths, the variations were not so marked.

In conclusion, I wish to express my gratitude to Dr. Paul C. Freer, Superintendent of Government Laboratories, for many valuable corrections of the chemical concepts put forth in this paper and for many helpful suggestions in its editing.

# DESCRIPTION OF PHOTOGRAPHS AND PHOTOMICROGRAPHS.[1]

FIG. 1. Showing the variation in the amount and type of liquefaction produced in forty-eight hours in gelatin of varying reaction and dryness. The +1.5 tube has not photographed well; it showed no liquefaction along the stab and but a small, circular liquefied depression at the surface.

2. Coverslip preparation from the human ileum showing the morphology of the organisms of culture "579" as seen scattered about among the desquamated epithelial cells. (The photograph has been carefully retouched by the photographer in order to aid reproduction.)

3. Culture "579," preparation from the edge of a +1 agar slant, grown for twenty-two hours at 35°–37°.

4. Culture "579," from just below the pellicle on +1 bouillon: twenty-four hours at 35°–37°.

5. Culture "579A," from the pellicle on +1 bouillon; twenty-four hours at 18°–25°.

6. Culture "579," from the pellicle formed on +1 gelatin; five days at 18°–25°.

7. Culture "Scout," from the *edge* of an eighteen-hour culture on +1 agar; at 35°–37°.

8. Culture "Scout," from the *center* of the same growth from which fig. 7 was prepared; showing older and involution forms.

9. Culture "561," from the *central* portion of a twenty-hour culture on +1 agar; at 35°–37°.

10. Culture "A," from the *central* portion of a twenty-hour culture on +1 agar; at 35°–37°.

11. Culture "city moat," from the *central* portion of a twenty-hour culture on +1 agar; at 35°–37°.

12. Culture "Pfeiffer," from the *central* portion of a twenty-hour culture on +1 agar; at 35°–37°.

[1] Taken by Charles Martin, photographer, Bureau of Government Laboratories. (X 880.)

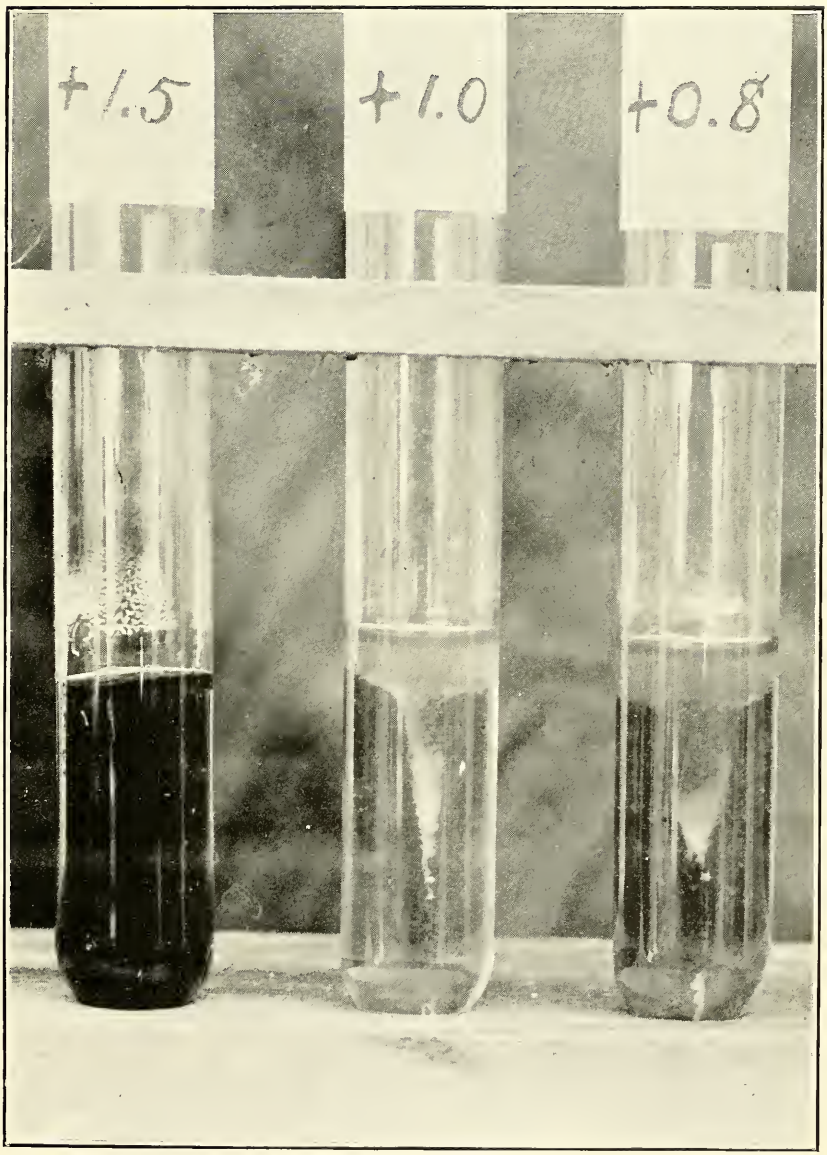


Photo by Martin.

FIG. 1.

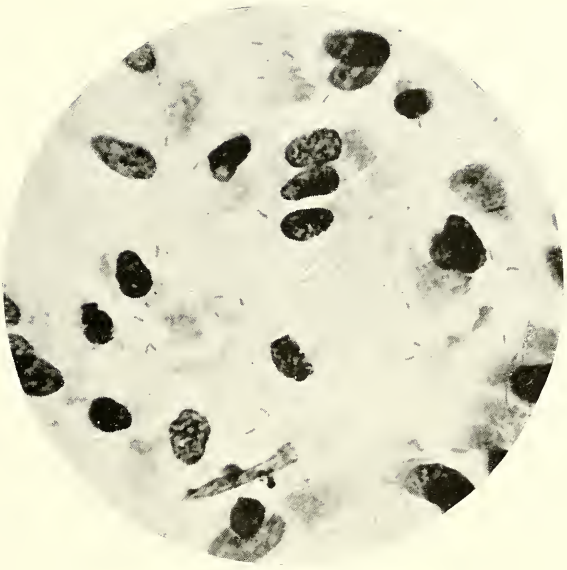

FIG. 2.

FIG. 3.

FIG. 4.

FIG. 5.

Fig. 6.

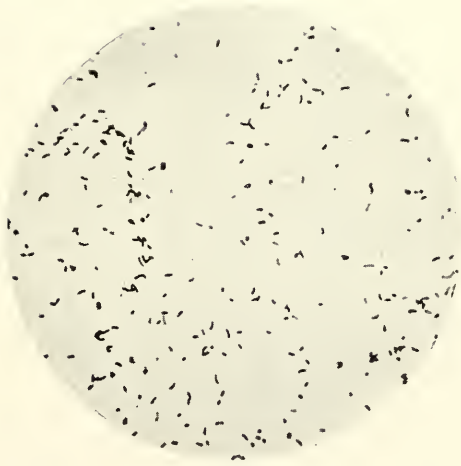

Fig. 7.

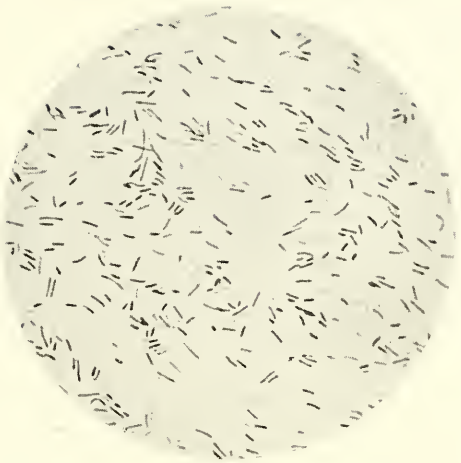

Fig. 8.

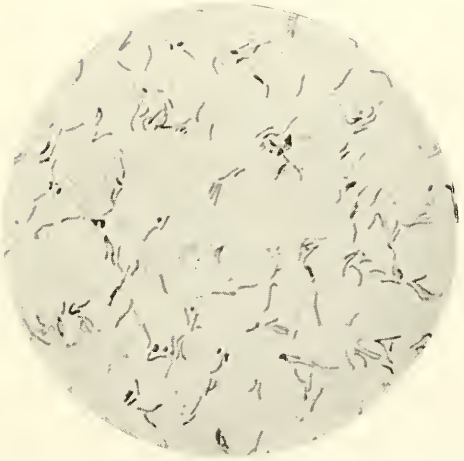

Fig. 9.

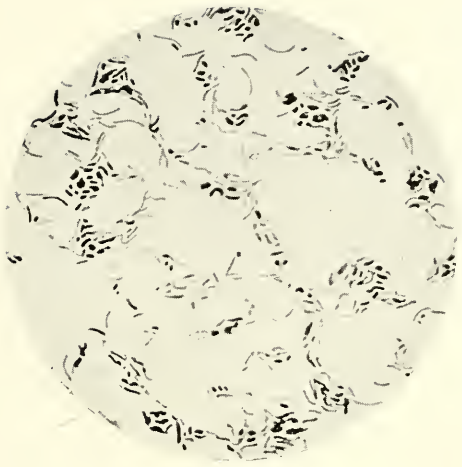

Fig. 10.

Fig. 11.

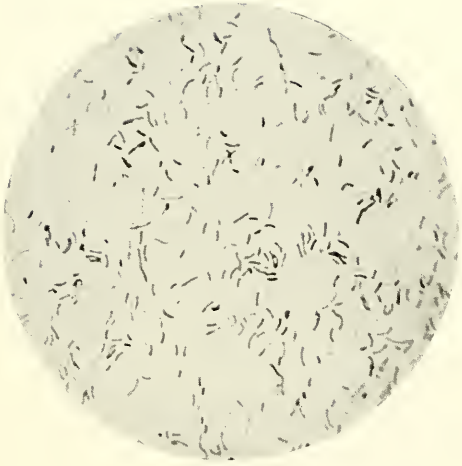

Fig. 12.